Biochemical Pharmacology, Vol. 43, No. 2, pp. 289–293, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

EFFECT OF ANTHELMINTICS ON THE ANTIOXIDANT SYSTEM OF *NIPPOSTRONGYLUS BRASILIENSIS*

JANMEJAI K. SRIVASTAVA,* SANJAY BATRA, SUMAN GUPTA,* JAGDISH C. KATIYAR* and VISHWA M. L. SRIVASTAVA†

Divisions of Biochemistry and *Parasitology, Central Drug Research Institute, Lucknow-226001, India

(*Received* 17 *May* 1991; *accepted* 9 *September* 1991)

Abstract—To understand the mode of anthelmintic action of thiabendazole and methyl-[5-[[4-(2-pyridinyl)-l-piperazinyl]carbonyl]-1*H*-benzimidazole-2-yl] carbamate (C.D.R.I. compound 81/470) against *Nippostrongylus brasiliensis*, their effect on the metabolism of reactive oxygen species in the parasite as well as in rat intestine was examined. Both drugs produced a significant depression in the levels of superoxide dismutase (SOD) and reduced glutathione (GSH) of the parasite. Release of antioxidant enzymes by the drug-treated worms was also found to be appreciably lowered. Both thiabendazole and compound 81/470 induced a depression in the levels of all five constituents of the antioxidant system of rat intestine but significant alterations were detected only in the GSH content of infected and the SOD activity of normal intestine. The production of O_2^- by treated intestine was, on the other hand, markedly enhanced. Increased formation of O_2^- by the host intestine accompanied with the reduced level of SOD and GSH in *N. brasiliensis* appear to have a deleterious effect on the parasite. Consequently, the drug-treated worms are unable to retain themselves *in situ* and are ultimately expelled. The greater effect produced on these parameters by thiabendazole compared to compound 81/470 is consistent with the relative efficacy of these anthelmintics.

Reactive oxygen species (ROs‡) and their scavengers play a vital role in establishment and rejection of helminthic infections [1, 2]. These species have been shown to exert a deleterious effect on almost all the parasites so far studied [3–6]. In order to become established in their hosts, helminth parasites employ two strategies: (1) they induce alterations in the host metabolism favouring lower production of ROs in the organ in which they reside [7, 8] and (2) the activity of their antioxidant enzymes like SOD, catalase and GPx [1, 2] is used. Depression in the activities of these enzymes on account of aging has been held responsible for the expulsion of *Nippostrongylus brasiliensis* from rat intestine 13 days post infection [9]. Likewise, the inhibition of catalase and GPx of *Acanthocheilonema viteae* by a potential macrofilaricidal agent has been established as the cause of damage leading ultimately to the death of the filariid [10].

Thiabendazole and the C.D.R.I. compound 81/470 show broad spectrum anthelmintic activity and reduce substantially the intensity of *N. brasiliensis* infection in rat [11, 12]. In an attempt to understand the mode of action of these anthelmintics, their effect on the ROs-metabolizing system of *N. brasiliensis* and rat intestine was examined.

MATERIALS AND METHODS

Infection and drug treatment. Male rats (Druckrey, 35–45 g) were divided into two groups. The animals of one group served as controls whereas those of the other group were infected by subcutaneous inoculation with 2000 ± 100 infective larvae of *N. brasiliensis* [13]. After 7 days each group of animals was further divided into three subgroups of which one was left untreated and the other two were treated orally with thiabendazole and compound 81/470, respectively. In order to recover *N. brasiliensis* in sufficient quantity, rats were administered with a subcurative dose (100 mg/kg body wt) and the worms were collected after 7 hr only. At this time, 33–38% of the worms had been expelled and most of the rest had migrated towards the posterior end of the intestine. The worms recovered from the intestine were motile but sluggish compared to the untreated parasites. After 24 hr, 91–93% of the worms were found cleared from the organ. Complete elimination of the nematode is achieved by three consecutive doses of 100 mg/kg of compound 81/470 or of 75 mg/kg of thiabendazole, or by a single dose of 150 mg/kg of either drug [11, 14, 15].

Four animals from each of the control subgroups were also killed following deep anaesthesia. The worms and intestine were cleaned thoroughly by repeated washings in normal saline.

Release of antioxidant enzymes. Worms (100–150 mg) were incubated at 37° in Hank's balanced salt solution containing 50 mM glucose. After 1 hr, the worms were removed and after centrifugation the medium was assayed for SOD [16], catalase [17] and GPx [18].

Release of O_2^- *and* H_2O_2. Rates of O_2^- and H_2O_2 release by the intestine were determined according to Nishikimi *et al.* [19] and Khan *et al.* [20], respectively. For each measurement, a 500 mg

† Corresponding author.

‡ Abbreviations: ROs, reactive oxygen species; SOD, superoxide dismutase; GPx, glutathione peroxidase; C.D.R.I. compound 81/470, methyl-[5-[[4-(2-pyridinyl)-1-piperazinyl]carbonyl]-1*H*-benzimidazole-2-yl] carbamate; GSH, reduced glutathione.

Table 1. Effect of anthelmintics on the antioxidant system of rat intestine

Antioxidant (units/mg protein)		Untreated activity	Thiabendazole		Compound 81/470	
			Activity	% Change*	Activity	% Change*
SOD†	Control	2.04 ± 0.15	1.09 ± 0.12	−46.6[b]	1.22 ± 0.14	−40.2[b]
	Infected	2.29 ± 0.17 (+12.3)[c]	1.63 ± 0.21	−28.8[c]	1.82 ± 0.19	−20.5[c]
Catalase‡	Control	0.231 ± 0.019	0.181 ± 0.012	−21.6[c]	0.166 ± 0.018	−28.1[c]
	Infected	0.249 ± 0.022 (+7.8)[c]	0.155 ± 0.014	−37.8[b]	0.178 ± 0.018	−28.5[c]
GPx§	Control	1.73 ± 0.21	1.28 ± 0.15	−26.0[c]	1.32 ± 0.27	−23.7[c]
	Infected	1.96 ± 0.24 (+11.8)[c]	1.44 ± 0.19	−26.5[c]	1.29 ± 0.19	−34.2[c]
GSH‖	Control	14.32 ± 1.07	10.41 ± 0.82	−27.3[c]	11.00 ± 0.67	−23.2[c]
	Infected	14.59 ± 1.02 (+1.9)[c]	7.41 ± 0.63	−49.2[a]	9.03 ± 0.66	−38.3[b]
Vitamin E‖	Control	0.055 ± 0.016	0.046 ± 0.011	−16.4[c]	0.062 ± 0.012	+12.7[c]
	Infected	0.066 ± 0.009 (+20.0)[c]	0.052 ± 0.006	−21.2[c]	0.063 ± 0.006	−4.5[c]

Values in parentheses denote the % change due to infection in the untreated group.
* Effect of anthelmintics (%) with respect to untreated controls.
† One unit is the amount that inhibits the auto-oxidation of epinephrine by 50%.
‡ μmol/min; § nmol/min; ‖ μg.
[a] $P < 0.005$; [b] $P < 0.05$; [c] $P > 0.05$.

Table 2. Drug-induced changes in the leakage of O_2^- and H_2O_2 by rat intestine

ROs		Untreated leakage	Thiabendazole		Compound 81/470	
			Leakage	% Change*	Leakage	% Change*
O_2^-	Control	21.55 ± 2.33	28.42 ± 1.26	+31.9[b]	23.82 ± 1.94	+10.5[c]
	Infected	18.75 ± 1.58 (−13.0)[c]	30.83 ± 1.29	+64.4[a]	23.33 ± 1.42	+24.4[b]
H_2O_2	Control	13.01 ± 0.40	15.17 ± 0.62	+16.6[b]	12.33 ± 0.51	−5.2[c]
	Intected	8.93 ± 1.06 (−31.4)[a]	10.09 ± 0.86	+12.9[c]	8.88 ± 0.63	−0.6[c]

Data presented as nmol/min/g tissue are means ± SE of four experiments.
Other details are the same as given in the legend to Table 1.

portion of the tissue was incubated for 30 min in phosphate-buffered saline pH 7.2 containing other reactants.

Oxygen uptake. Rate of oxygen consumption by *N. brasiliensis* and rat intestine was measured polarographically in the closed system of a Gilson Oxygraph, model 5/6 H equipped with a recorder. A 15–20 mg portion of the tissue or the parasite was introduced into the assay chamber containing 1.6 mL of air-saturated Hank's balanced salt solution and equilibrated for 3 min. The electrode chamber was then closed and uptake rate assayed [21].

Preparation of homogenate and determination of the levels of antioxidants (enzymatic and nonenzymatic). Remaining portions of the intestine as well as of the worms were homogenized in isotonic KCl to the strength of 10% and 5% (w/v), respectively. The homogenate was centrifuged at 900 *g* × 10 min followed by recentrifugation at 9000 *g* × 30 min. Supernatants were assayed for SOD [16, 22], catalase [17], GPx [18], GSH [23] and vitamin E [24]. Protein content was measured colorimetrically using bovine serum albumin as a standard [25].

Chemicals. Glutathione reductase, horseradish peroxidase, SOD, Phenol red, NADPH and NADH were procured from the Sigma Chemical Co. (St Louis, MO, U.S.A.). GSH, nitroblue tetrazolium and phenazine methosulphate were purchased from SISCO Research Laboratories, Bombay, India.

Epinephrine and bathophenanthroline were the products of Romali and Loba Chemie, Bombay, respectively. All other chemicals were of analytical grade. Thiabendazole was obtained as a gift from Merck, Sharp and Dohme (India) Ltd, Bombay, while compound 81/470 was prepared by the Medicinal Chemistry Division of the Institute.

RESULTS

N. brasiliensis infection on day 7 did not cause any noticeable change either in the activities of antioxidant enzymes or in the levels of GSH and vitamin E in rat intestine. The increases recorded in these parameters, with the maximum being of 20% in vitamin E content, were statistically insignificant (Table 1). Treatment of the animals with thiabendazole or compound 81/470, on the contrary, lowered the levels of all five antioxidants both in normal and infected intestine. Maximum depression in the control tissue was recorded for SOD whereas in the infected tissue it was for GSH. These changes were also significant statistically. Thiabendazole induced alterations in most of the constituents greater than those induced by compound 81/470.

Nematode infection reduced the leakage of both O_2^- and H_2O_2 by the intestine but of the latter species highly significantly (Table 2). Thiabendazole, on the contrary, enhanced appreciably the release of O_2^- by normal as well as by infected intestine. Compound 81/470, surprisingly, stimulated the release of only O_2^- and that too by the infected as well as the normal tissue.

Thiabendazole and compound 81/470 impaired the antioxidant system of *N. brasiliensis* also (Fig. 1a–c). The nematode isolated from rat after 7 hr of treatment with the drugs exhibited reduced levels of all the five scavengers examined. The decline observed in SOD and GSH content was highly significant while that recorded in catalase activity was insignificant. The effectiveness of thiabendazole was always greater except in the case of GPx and vitamin E, where compound 81/470 exhibited a greater and significant depression.

The treated parasites, in addition, expressed retarded secretion of the antioxidant enzymes into the ambient medium (Fig. 2). Here again thiabendazole was found to be more effective. Both thiabendazole and compound 81/470 reduced markedly the O_2 consumption capacity of *N. brasiliensis* and, in contrast, enhanced uptake by the intestine (Fig. 3).

DISCUSSION

In vitro studies with artifical systems have established that ROs are injurious to all helminth parasites studied irrespective of their developmental forms or the site of predilection. The parasites maintain themselves *in situ* by inactivating host-generated oxidants through their antoxidant enzymes and other scavengers [1, 2, 9, 10, 26]. Also a few parasites have been shown to modulate host metabolism in favour of lower accumulation of ROs in the tissue in which they dwell [7, 8].

N. brasiliensis, the rat intestinal nematode, is also

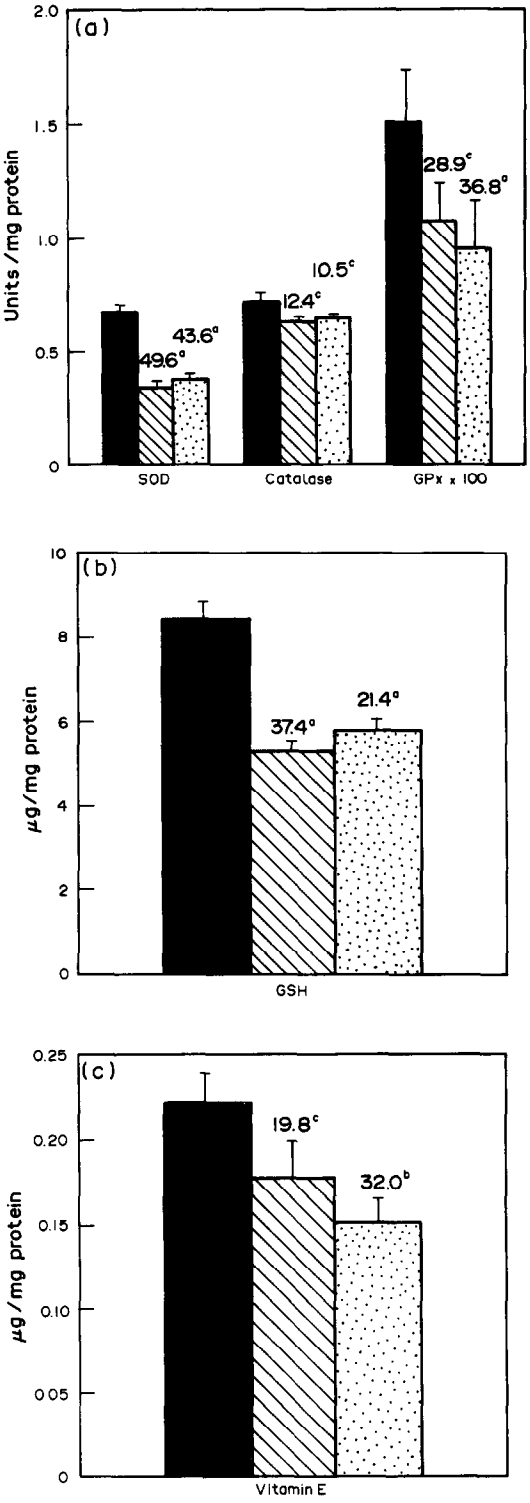

Fig. 1. Drug-induced alterations in the antioxidant system of *N. brasiliensis*. (a) antioxidant enzymes, (b) reduced glutathione, (c) vitamin E. (■) Control, (▨) thiabendazole, (▩) compound 81/470. One unit of SOD corresponds to the amount of protein which inhibits the reduction of nitroblue tetrazolium by 50%; one unit of catalase = µmol/min and one unit of GPx = nmol/min. Values above the bar denote percentage change with respect to control where (a) $P < 0.005$; (b) $P < 0.05$; (c) $P > 0.05$. Data are means of four determinations.

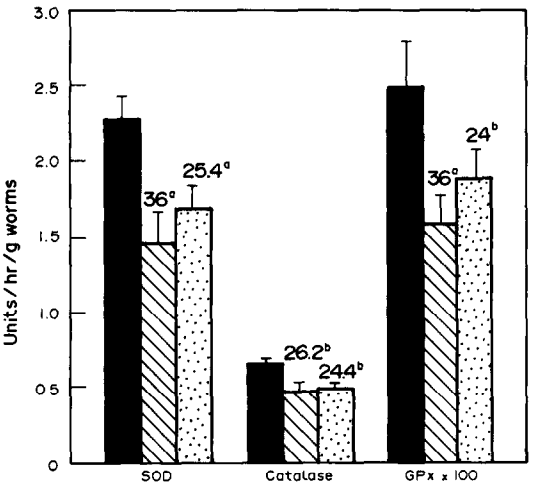

Fig. 2. Drug-induced alterations in the release of antioxidant enzymes by *N. brasiliensis*. Details are the same as given in the legend to Fig. 1.

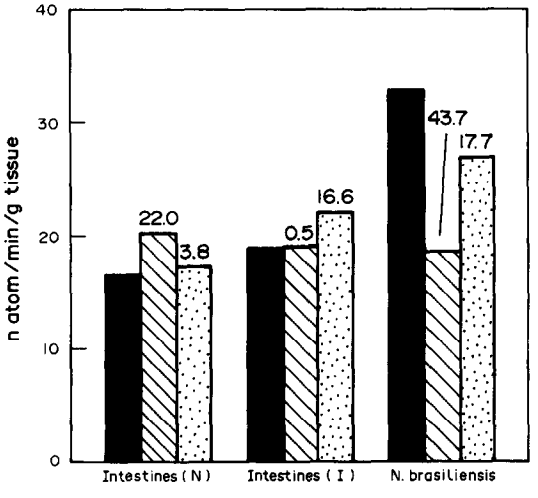

Fig. 3. Drug-induced alterations in oxygen uptake by rat intestine and *N. brasiliensis*. Data are means of two determinations. Other details are the same as given in the legend to Fig. 2.

sensitive to ROs and possesses major antioxidant enzymes, namely SOD, catalase, GPx and peroxidase, in substantial activities [26, 27]. The results of the present study indicate that infection with this parasite on day 7 does not introduce any significant alteration in the ROs-scavenging system of the rat intestine (Table 1). However, decreased release of ROs, H_2O_2 in particular, would appear to facilitate the establishment of the parasite in the host intestine. The nematode *per se* secretes SOD, catalase and GPx and thereby detoxifies ROs present in its immediate surroundings (Fig. 2).

Thiabendazole and compound 81/470 disrupt this compromise between the host and the parasite. At the host level, these compounds enhance appreciably the leakage of O_2^- into the lumen (Table 2). In the case of *N. brasiliensis*, on the other hand, the drugs reduce appreciably the content as well as the leakage of the antioxidant enzymes (Figs 1a and 2). Together, these changes appear to severely damage the parasite. The depressed levels of GSH and vitamin E further deprive the nematode of its capacity to protect itself against the injurious effects of ROs (Fig. 1b and c). It is pertinent to mention that similar types of change recorded in intestine and the worms 13 days post infection have been considered responsible for clearance of the parasites at this stage [9].

The damaged nematode loses its capacity to consume O_2 (Fig. 3). Consequently, carbohydrate metabolism is shifted towards the anaerobic side leading to a sharp decline in ATP synthesis [28]. In brief, ROs toxicity seems to impair a variety of physiological functions in *N. brasiliensis* as a result of which the parasite cannot maintain itself *in situ* and is ultimately expelled by the host. The greater effect on most of the activities produced by thiabendazole than compound 81/470 is in good accord with the higher chemotherapeutic efficacy of the former drug [11, 12].

Interestingly, both thiabendazole and compound 81/470 belong to the benzimidazole family the members of which are known to disrupt microtubules by virtue of their interaction with tubulin [29]. Hence, the effects observed in the present study on the ROs-metabolizing system should not be treated as the primary or the only mode of action. Nevertheless, interference with the antioxidant system may be more important because this can alter directly the structure and functions of numerous biomolecules including tubulin *per se*.

Surprisingly, both the anthelmintics impair ROs metabolism in the control intestines too (Table 1). Amongst these, the substantial depression recorded in SOD activity is of particular interest, indicating drug toxicity to the host. However, since the levels of vitamin E and GSH are not lowered significantly, the chances of tissue damage at this dose level seem small. At the LD_{50} dose in rat for thiabendazole and compound 81/470 which is 3.6 and 2.15 g/kg, respectively [15, 30], the alterations in the ROs-metabolizing system are expected to be more pronounced and may account for drug toxicity.

Acknowledgements—The authors S.B. and J.K.S. are grateful to the Council of Scientific and Industrial Research, New Delhi, for the award of Senior Research Fellowship and Research Associateship, respectively.

REFERENCES

1. Callahan HL, Crouch RK and James ER, Helminth antioxidant enzymes: a protective mechanism against host oxidants? *Parasitol Today* **4**: 219–229, 1988.
2. Srivastava VML and Batra S, Role of oxygen derived species in establishment and pathology of nematode parasites. In: *Perspectives in Nematode Physiology and Biochemistry* (Ed. Sood ML). Narendra Publishing House, Delhi.
3. Callahan HL, James ER and Crouch RK, Hydrogen

peroxide is the toxic xanthine oxidase product for *Onchocerca microfilariae*. *J Cell Biol* **107**: 3523–3532, 1988.
4. Rzepczyk CM, Bishop CJ, Cheung K, Atwell R and Ferrante A, Stimulation of neutrophil respiratory burst and iodination reaction by opsonized microfilariae of *Dirofilaria immitis*. *Aust J Exp Biol Med Sci* **64**: 43–51, 1986.
5. Bass DA And Szejda P, Mechanisms of killing of newborn larvae of *Trinchinella spiralis* by neutrophils and eosinophils. *J Clin Invest* **64**: 1558–1564, 1979.
6. Murray HW, Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages. *J Exp Med* **153**: 1302–1315, 1981.
7. Batra S, Singh SP, Srivastava VML and Chatterjee RK, Xanthine oxidase, superoxide dismutase, catalase and lipid peroxidation in *Mastomys natalensis*: effect of *Dipetalonema viteae* infection. *Ind J Exp Biol* **27**: 1067–1070, 1989.
8. Singh SP, Batra S, Gupta S, Katiyar JC and Srivastava VML, Effect of *Ancylostoma ceylanicum* infection in hamsters on enzymes that metabolize reactive oxygen intermediates. *Med Sci Res* **17**: 493–495, 1989.
9. Batra S, Srivastava JK, Gupta S, Katiyar JC and Srivastava VML, Role of reactive oxygen species in expulsion of *Nippostrongylus brasiliensis* from rat. *Parasitology*, submitted.
10. Batra S, Chatterjee RK and Srivastava VML, Antioxidant enzymes in *Acanthocheilonema viteae* and effect of antifilarial agents. *Biochem Pharmacol* **40**: 2363–2369, 1990.
11. Katiyar JC, Misra A, Gupta S, Visen PKS, Murthy PK and Sen AB, Efficacy of a substituted methyl benzimidazole carbamate against developing adult helminth parasites. *Vet Parasitol* **23**: 193–204, 1987.
12. Misra A, Katiyar JC and Sen AB, Experimental studies with *Nippostrongylus brasiliensis* on factors modifying therapeutic efficacy of anthelmintics in rats. *Ind J Exp Biol* **18**: 906–909, 1980.
13. Agarwal A, Misra SK, Misra A, Katiyar JC and Ghatak S, The effect of levamisol on the level of biogenic amines in *Nippostrongylus brasiliensis*. *Ind J Med Res* **78**: 651–655, 1983.
14. Misra A, Visen PKS and Katiyar JC, Comparative efficacy of standard antihookworm drugs against various test nematodes. *J Helminth* **55**: 273–278, 1981.
15. Katiyar JC, Visen PKS, Misra A, Gupta S and Bhaduri AP, Methyl 5(6)-4,2-pyridyl piperazino carbamoyl benzimidazole-2-carbamate, a new broad spectrum anthelmintic. *Acta Tropica* **41**: 279–286, 1984.
16. Beauchamp C and Fridovich I, Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* **44**: 276–287, 1971.
17. Aebi H, Catalase *in vitro*. In: *Methods in Enzymology* (Ed. Packer L), Vol. 105, pp. 121–126. Academic Press, New York, 1984.
18. Leopold F and Wolfgang AG, Assays of glutathione peroxidase. In: *Methods in Enzymology* (Ed. Packer L), Vol. 105, pp. 114–121. Academic Press, New York, 1984.
19. Nishikimi M, Rao AN and Yagi K, The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem Biophys Res Commun* **46**: 849–854, 1972.
20. Khan SH, Emerit I and Feingold J, Superoxide and hydrogen peroxide production by macrophage of New Zealand black mice. *Free Rad Biol Med* **8**: 339–345, 1990.
21. Srivastava VML, Agarwal N, Visen PKS and Katiyar JC, *Ancylostoma ceylanicum*: ATP production and effect of anthelmintics. *Ind J Exp Biol* **28**: 578–581, 1990.
22. Misra HP and Fridorich I, The univalent reduction of oxygen by reduced flavins and quinones. *J Biol Chem* **247**: 188–192, 1972.
23. Beutter E, Duron O and Kelly BM, Improved method for the determination of blood glutathione. *J Lab Clin Med* **61**: 882–888, 1963.
24. Desai ID, Vitamin E analysis methods for animal tissues. In: *Methods in Enzymology* (Ed. Packer L), Vol. 105, pp. 138–147. Academic Press, New York, 1984.
25. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
26. Batra S, Singh SP, Gupta S, Katiyar JC and Srivastava VML, Reactive oxygen intermediates metabolizing enzymes in *Ancylostoma ceylanicum* and *Nippostrongylus brasiliensis*. *Free Rad Biol Med* **8**: 271–274, 1990.
27. Smith NC and Bryant C, The role of host generated free radicals in helminth infections: *Nippostrongylus brasiliensis* and *Nematospiroides dubius* compared. *Int J Parasitol* **16**: 617–622, 1986.
28. Srivastava JK, Gupta S, Katiyar JC and Srivastava VML, Effects of methyl[5[[4-(2-pyridinyl)-1-piperazinyl]carbonyl]-1*H*-benzimidazol-2-yl] carbamate on energy metabolism of *Ancylostoma ceylanicum* and *Nippostrongylus brasiliensis*. *Ind J Exp Biol* **27**: 735–738, 1989.
29. Van den Bossche H, Rochette F and Horig C, Mebendazole and related anthelmintics. *Adv Pharmacol Chemother* **19**: 67–128, 1982.
30. Cavier R, Chemotherapy of intestinal nematodes. In: *Chemotherapy of Helminthiasis, International Encyclopedia of Pharmacology and Therapeutics* (Eds. Cavier R and Hawking F), pp. 215–436. Pergamon Press, New York, 1973.